

TABLE 3

| | G1 | G2 | G3 | G4 | G5 | G6 |
|----------------|--------------------------------|-------------------------------------|--|--|------------------------|----------|
| Subject/Result | PS06B-CRM122 (R: 1/1, PS 84 | PS06B-CRM123 (R: 3/1, PS 84 kDa) | PS06B-CRM124 (R: 1.5/1, PS 350 kDa) | PS06B-CRM125 (R: 2.9/1, PS 350 kDa) | PS06B-CRM003 (CDAP) | PS06B-PD |
| GMC (UG-ML) | 0.83 | 0.37 | 1.18 | 0.64 | 0.31 | 0.10 |
| Responders(%) | 31/40 | 26/40 | 33/40 | 29/40 | 29/40 | 15/40 |

The immunogenicity of these conjugates in balb/c mice is described in FIG. 3. Together FIG. 3, and table 3 demonstrate that in the mouse model the conjugates produced by reductive amination were comparable with those produced using CDAP chemistry. In particular FIG. 3 demonstrates that the immunogenicities of the conjugates produced using reductive amination was higher than the immunogenicity of the conjugate made using CDAP chemistry. Table 4 Describes the GMC Levels Obtained by Immunisation of Guinea Pigs with the Conjugates Made Using the Methods of Example 4.

TABLE 4

| | G1 | G2 | G3 | G4 | G5 | G6 |
|----------------|-------------------------------------|------------------------------------|--|--|------------------------|----------|
| Subject/Result | PS06B-CRM122 (R: 1/1, PS 84 kDa) | PS06B-CRM123 (R: 3/1, PS84 kDa) | PS06B-CRM124 (R: 1.5/1, PS0350 kDa) | PS06B-CRM125 (R: 2.9/1, PS 350 kDa) | PS06B-CRM003 (CDAP) | PS06B-PD |
| GMC (UG-ML) | 3.51 | 7.70 | 2.84 | 19.93 | 3.70 | 1.55 |
| Responders(%) | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 |

The immunogenicity of these conjugates in guinea pigs is described in FIG. 4. Similar to the experiments carried out in the mouse model, the results in table 4 and FIG. 4 show that the conjugates produced by reductive amination were comparable with those produced using CDAP chemistry, in particular PS06B-CRM125 demonstrated significantly higher GMC levels and immunogenicities than the conjugate produced using CDAP.

Example 7

Conjugation of Hib to Tetanus Toxoid Using Reductive Amination

Hib-104-LS080

2.9 g of PS (orcinol dosage, AHIBCPA007 lot) were dissolved in 260 ml of 10 mM phosphate buffer (Na/K₂) pH 6.2 for 4 h30 at room temperature and then overnight at +4° C. The viscosity follow-up was done during the dissolution. After 4 hours dissolution the viscosity seemed to be stable. PS was diluted at 10 mg/ml with phosphate buffer and then oxidised in the dark with 0.07 molar equivalent of NaO₄ during 60 minutes. Oxidised PS was diafiltered (Sartorius Hydrosart 2 kDa) against 3.5 volumes of phosphate buffer and then filtered on a 0.22 µm filter. The number of repeating units obtained after oxidation was estimated by ¹H-NMR and was found to be around 21.

Hib-TT-LS210, 212 and 213

200 mg of oxidised PS (14.56 mg/ml) were mixed with 300 mg of TT (31.18 mg/ml, TT/PS ratio (w/w): 1.5/1) and diluted to 4 mg/ml with 36.64 ml of 10 mM phosphate buffer (Na/K₂) pH 6.2. The solution was lyophilized in the presence of a stabilising agent. Lyophilized PS+TT was solubilised with 20 ml of DMSO for 6 hrs at 25° C. Then 10 Meq of TAB (Sodium triacetoxymethylborohydride) were added (38.7 mg) and after 16 hrs

under agitation, 2 molar equivalent of NaBH₄ (100 mg/ml in 0.1M NaOH) was added followed by an incubation for 30 min at room temperature. The solution was diluted 3× by addition of WFI followed by a diafiltration step (5 volumes of WFI followed by 5 volumes of 10 mM acetate buffer 150 mM NaCl pH 6.2, 100 kDa MWCO). The sample was then loaded on Sephacryl S300HR resin. Elution was carried out in 10 mM acetate buffer using 150 mM NaCl (pH 6.2). Interesting fractions were pooled and filtered on a 0.22 µm filter. The resulting conjugates had a final TT/PS ratio (w/w) of 2.1/1.

The invention claimed is:

1. A process for conjugating a bacterial saccharide and reducing the sizing effect on bacterial saccharide comprising the steps of

- a) reacting the bacterial saccharide with 0.001-0.7 molar equivalents of periodate to form an activated bacterial saccharide;
 - b) mixing the activated bacterial saccharide with a carrier protein;
 - c) reacting the activated bacterial saccharide and the carrier protein with a reducing agent to form a conjugate;
- wherein step a) occurs in a buffer which does not contain an amine group, and the buffer has a concentration between 1-100 mM and wherein the bacterial saccharide is *S. pneumoniae* capsular saccharide 6B.

2. The process of claim 1 wherein the buffer is selected from the group consisting of phosphate buffer, borate buffer, acetate buffer, carbonate buffer and citrate buffer.

3. The process of claim 1 wherein the pH in step a) is pH 3.5-8.0.

4. The process of claim 1 wherein the average molecular weight of the bacterial saccharide is between 1-1100 kDa after step a).

5. The process of claim 1 wherein the carrier protein is selected from the group consisting of tetanus toxoid, fragment C of tetanus toxoid, diphtheria toxoid, CRM197, Pneumolysin, protein D, PhtD, PhtDE and N19.

6. The process of claim 1 wherein the reducing agent comprises sodium cyanoborohydride or sodium triacetoxymethylborohydride.

7. The process of claim 1 comprising a further step e) of purifying the conjugate.

8. The process of claim 1 containing a further step of mixing the conjugate with further antigens.

9. The process of claim 8 wherein the further antigens comprise one or more *S. pneumoniae* proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX trun-